ANTIBACTERIAL CHARACTERISTICS OF FISH
PROTAMINES. VIII¹
EFFECT OF CLUPEINE SULFATE ON THE MEM-
BRANE-ATPase ACTIVITY AND MEMBRANE
STRUCTURE OF *BACILLUS SUBTILIS*

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(Received July 14, 1987)

We studied the effect of clupeine sulfate on a vital membrane-bound enzyme (adenosinetriphosphatase or ATPase). Clupeine stimulated the Mg^{2+} -ATPase activity to 250% of the control level but the stimulatory effect diminished with more than 200 μg of clupeine/ml. Considering the vital metabolic functions of this enzyme it appears that the stimulatory effect of clupeine on this enzyme may contribute to the destruction of membrane function. Phosphatidylethanolamine, a typical membrane phospholipid inactivated and blocked the stimulatory effect of clupeine on the ATPase activity.

In view of the strong interaction between clupeine and membrane lipids, the effect of clupeine on the membrane structure of *Bacillus subtilis* was studied by incorporating a hydrophobic fluorescent dye (1,6-diphenyl-1,3,5-hexatriene) molecule in the isolated cell membranes. Clupeine increased the fluorescence intensity and decreased the fluorescence polarization of the membrane dye environment. This indicated a structural change in the cell membrane.

In an accompanying paper (1), and in a previous paper (2) we have reported that clupeine sulfate (a fish protamine) strongly interacts with the cytoplasmic membrane of *Bacillus subtilis*. The site of interaction was identified as the membrane

¹ Abbreviations used in this text are as follows: ATPase, adenosinetriphosphatase; DPH, 1,6-diphenyl-1,3,5-hexatriene; CTAB, cetyltrimethylammonium bromide.

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phospholipids and this interaction leads to the release of cytoplasmic constituents, lysis of protoplasts, destruction of the membrane function and finally the death of the cells.

The present investigation was carried out to learn more about the cause of the loss of membrane function by studying the effect of clupeine on an important membrane-bound enzyme. In addition, the consequences of clupeine-membrane interaction on the structure of the membrane was also studied by monitoring the hydrophobicity and fluidity of the membrane lipids by using a fluorescent probe incorporated in the cell membrane.

The enzyme ATPase of *B. subtilis* was chosen for this study because ATPases are usually associated with biological membranes and known to be implicated in many cellular functions(3). Properties of this enzyme in several microbial species have been described previously (4–8).

MATERIALS AND METHODS

Organism growth condition, and preparation of cell membrane. All were the same as described in the preceding paper (1).

Chemicals. The clupeine sulfate used was the same preparation as described in the preceding paper(1). Adenosinetriphosphate (ATP, Na-salt), 1,6-diphenyl-1,3,5-hexatriene (DPH), phosphatidylethanolamine, polymyxin B, sodium deoxycholate and cetyltrimethylammonium bromide (CTAB) were obtained from Sigma Ltd. All other chemicals were of reagent grade.

ATPase assay. ATPase activity was measured by a modification of the method of ROSENTHAL and MATHESON (6). The assay mixture consisted of 2.5 mM ATP, 1.25 mM MgCl_2 or CaCl_2 , 75 mM Tris-HCl buffer, pH 8.0, approximately 0.25–0.3 mg of membrane protein as determined by the method of LOWRY et al. (10). Clupeine at desired concentration was added to the assay mixture when the influence of clupeine was measured. The total volume of assay mixture was 2.0 ml and incubated at 37°C. After 15–20 min, the reaction was terminated by making the mixture 2.5% in cold HClO_4 . The tubes were centrifuged for 5 min to remove precipitated protein and up to 1.2 ml aliquots were removed for the analysis of P_i (orthophosphate). The liberation of P_i was estimated by the method of GLASER and HOFER (8). To the 1.2 ml of assay mixture to be analyzed for P_i , 2.0 ml of a solution containing 2.0% (v/v) H_2SO_4 , 0.5% (w/v) SDS and 0.5% (w/v) ammonium molybdate, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ was added. After adding 20 μl of 10% (w/v) ascorbic acid the mixture was incubated for 10 min at 37°C. The absorbance was then measured at 750 nm in a Hitachi 228A spectrophotometer. The analytical values were corrected for the non-enzymatic hydrolysis of the substrate and for the contents of extraneous P_i in the enzyme preparation and in the reagents.

Fluorescence measurements of clupeine-treated cell membranes. Cytoplasmic membranes isolated from *Bacillus subtilis* were suspended in 0.15 M KCl and used immediately for fluorescence studies. The fluorescent hydrocarbon 1,6-diphenyl-

1,3,5-hexatriene (DPH) was used as a probe for monitoring hydrophobicity and fluidity of the cell membrane. Labeling was done as described by SHINITZKY and INBAR (9). For labeling, membrane suspensions were treated with an equal volume of 2 μM DPH in 0.15 M KCl. Membrane samples prepared similarly without DPH were used as scatter controls. The binding of DPH with cell membrane was indicated by a steep increase in fluorescence intensity, which was stabilized after about 60 min at room temperature (25°C). The membranes were pelleted at 25,000 g for 20 min and then suspended in 0.15 M KCl. These suspensions were immediately used for fluorescence measurements.

The DPH-labeled membranes were treated with different concentrations of clupeine or buffer for 15 min at 25°C. Fluorescence was monitored with a Hitachi Perkin-Elmer MPF-3 spectrofluorometer. The excitation and emission monochrometers were set at 366 nm and 430 nm respectively. The excitation beam was obtained with a polarizer. Fluorescence was detected in two cross-polarized channels following passage through 2 M aqueous NaNO_3 to cut off scattered light below 390 nm. Fluorescence polarization and intensity were obtained by simultaneously measuring the fluorescence intensity parallel (I_{ii}) and perpendicular (I_i) to the polarized exciting beam. These values relate to the degree of fluorescence intensity (F) and polarization (P) by the following equations (9):

$$F = I_i (I_i/I_{ii}) + 2$$

$$P = \frac{(I_{ii}/I_i) - 1}{(I_{ii}/I_i) + 1}$$

Temperature was maintained at 25°C throughout. All values were corrected for controls.

RESULTS

Properties of membrane-bound ATPase of B. subtilis

Maximum ATPase activity in the isolated membranes was observed at pH 8.0 and the enzyme activity was dependent on the divalent cations Mg^{2+} or Ca^{2+} . ATPase activities remained unchanged for 60 days when membranes were suspended in 50 mM Tris-HCl buffer at pH 8.0 and stored at 4°C, but activity was abolished by heating at 60°C and freezing at -20°C followed by thawing at 25°C.

Influence of clupeine on the membrane-bound Mg^{2+} -ATPase activity

Figure 1 shows the effect of various concentrations of clupeine on the Mg^{2+} -ATPase activity. Clupeine was found to stimulate the enzyme activity by 250% of the control level. The Mg^{2+} -ATPase activity in the membrane preparation was gradually stimulated and reached the maximum at 150–200 μg of clupeine/ml. At the concentrations higher than 200 μg /ml the rate of stimulation decreased and finally leveled off at 300–400 μg /ml. This result indicated that an optimum

Fig. 1.

Fig. 2.

Fig. 1. Stimulatory effects of different concentrations of clupeine on the membrane-bound Mg^{2+} -ATPase activity of *B. subtilis*. Control enzyme activity is the activity without clupeine.

Fig. 2. Inactivation by phosphatidylethanolamine of the stimulatory influences of various concentrations of clupeine on the membrane-ATPase activity of *B. subtilis*. ▲: phospholipid alone, △: with clupeine 50 $\mu g/ml$, ●: with clupeine 100 $\mu g/ml$, ○: with clupeine 150 $\mu g/ml$.

concentration of clupeine was necessary for the maximum stimulation of ATPase activity. Addition of clupeine up to 400 $\mu g/ml$ had no effect on the pH of the assay mixture.

Comparison of the effects of clupeine and some other membrane-active agents on the membranes and enzyme activity

The data presented in Table 1 shows the comparative effect of membrane-active agents on the stimulation of ATPase activity and release of enzyme activity from the membranes. Sodium deoxycholate stimulated ATPase activity, accompanied by the disruption of the membrane as examined by phase contrast microscope. A significant amount of enzyme activity was also released from the membrane. Stimulation of enzyme activity was not accompanied by the release of enzyme activity when the membranes were treated with either clupeine, polymyxin B or CTAB, but clumps or aggregation of the membranes were observed which may be due to strong interaction between the cationic agents and the membranes.

Influence of a membrane phospholipid on the stimulation of membrane-ATPase activity by clupeine

In the preceding paper (1) we reported that clupeine binds with membrane phospholipids. To determine whether phospholipids can antagonize the stimulatory effect of clupeine on ATPase activity, measured amounts of clupeine were mixed

Table 1. Comparison of the stimulation of ATPase activity and release of enzyme activity by various membrane-active agents.^a

Agent	Concentration	Stimulation of enzyme activity ^b (%)	Release of enzyme activity ^b (%)
Sodium deoxycholate	0.05%	256	180
Polymyxin B	100 µg/mg membrane protein	170	0
CTAB	1.0 mg/mg membrane protein	210	0
Clupeine	200 µg/mg membrane protein	185	0

^a Membranes were mixed with each agent and incubated for 20 min in 0.1 M Tris-HCl buffer, pH 7.2. A portion of the mixture was assayed for ATPase activity and another portion was centrifuged at 30,000 *g* and the supernatant was assayed for released enzyme activity.

^b Percent of control activity.

with different concentrations of phosphatidylethanolamine and the membranes were mixed with these mixtures and assayed for the ATPase activity. Figure 2 shows that stimulation of ATPase activity by 50, 100 and 150 µg/ml of clupeine were blocked by the phospholipid at concentrations 5–15 µg/ml, and ATPase activity decreased to around control levels. The concentrations at which phospholipids blocked the effect of clupeine on ATPase were close to the molar equivalent of clupeine in the assay mixture. Phospholipid added alone had very little influence on the ATPase activity. This results suggested that anionic phospholipids external to the membrane reacted with polycationic clupeine in molar proportions and this action probably resulted in the neutralization of charges. If this is true, clupeine is not able to react with the anionic targets in the cell membrane. This reaction appears to be essential for the effects on membrane-bound ATPase activity.

Effect of clupeine on DPH-labeled membrane fluorescence intensity and polarization

The changes in the fluorescence intensity and polarization of a hydrophobic dye incorporated in the isolated cell membranes can give information concerning the hydrophobicity and fluidity of the dye environment which are directly linked to the structural integrity of the cytoplasmic membrane (9, 14). The fluorescent dye 1,6-diphenyl-1,3,5-hexatriene (DPH), an asymmetric hydrophobic molecule, was used as a fluorescent probe. As shown in Fig. 3, the fluorescence polarization values of DPH-labeled membranes gradually decreased with the addition of clupeine up to 15 µg/ml and became stable with higher concentrations. On the other hand the fluorescence intensity of DPH-labeled membranes increased sharply with clupeine concentration up to 15 µg/ml and then decreased abruptly with higher concentrations.

This increase in fluorescence intensity observed with the addition of clupeine

Fig. 3. Effect of clupeine on the fluorescence intensity (○), and fluorescence polarization (●) of *B. subtilis* cytoplasmic membrane prelabeled with a fluorescent hydrophobic dye molecule (DPH).

was directly proportional to the change in the fluorescent life-time of the dye-excited state, indicating an increase in relative hydrophobicity of the dye environment of the membrane. The fluorescence polarization data would indicate a decrease in membrane fluidity. It is likely that this change would adversely affect the liquid crystalline state of the membrane lipids and cause detectable structural changes in the cytoplasmic membrane.

DISCUSSION

The properties of the membrane-bound ATPase of the *B. subtilis* used in this study were more or less similar to ATPases from other sources, particularly those from various *Bacillus* organisms (4–6).

ATPases are quite universally associated with biological membranes and participate in the energy transfer and transformation of these organelles (3). Due to such vital functions of membrane-ATPase, it is conceivable that any external influence on ATPase, either stimulation or inhibition, could be lethal. The actual mechanism of stimulation of ATPase activity by clupeine is not clearly understood. However, the highly cationic nature of clupeine may be indirectly responsible for the stimulation because binding of clupeine with the membrane phospholipids may change the membranous environment for the normal activity of the enzyme. However, enhanced ATPase activity could be a manifestation of a shift in the equilibrium of the reaction *in vivo* to a more unfavourable state for the synthesis of ATP and ultimately affecting the cellular metabolic activities. Further, stimulation of the hydrolytic activity of the enzyme would deplete the store of available ATP

required by the cell for ion transport, therefore, translocation of other nutrients which are secondarily driven by this enzyme through the gradient established between Na^+ and K^+ would be deranged and leakage of important solutes would occur. In a previous report(2) we have already reported that clupeine induces release of 260 nm material, inorganic phosphate and pentose. Results of the present study suggested the possibility of a two-way mechanism for the bactericidal action of clupeine: one by clupeine-membrane interaction causing the damage to the membrane which has already been described in the previous paper(1) and secondarily by stimulation of the ATPase activity. It is possible that these two phenomena are inseparable, that one cannot occur without the other.

The solubilization of the membrane-ATPase by sodium deoxycholate and the failure of clupeine, CTAB and polymyxin B to do so (Table 1) are consistent with the previous findings of the action of these agents on protoplasts and isolated membranes(1, 3, 11-13). HAROLD pointed out that several basic peptides and polypeptide antibiotics react with phospholipid moieties of membranes reorienting them to produce discontinuities and channels in the osmotic barrier (3). Inactivation of clupeine action on ATPase activity by phosphatidylethanolamine external to the cell membrane proved that it is essential for clupeine to react with the membrane lipids for it to act on ATPase or on the bacteria as a whole. Our present studies on the fluorescence intensity and polarization of DPH-labeled isolated membranes showed that clupeine considerably affect the membrane structure apparently by changing the hydrophobicity and fluidity of the membrane lipid layer. Although it is difficult to interpret all these data in molecular terms, the strong affinity of clupeine for phospholipids would certainly be enough evidence for such actions. It is therefore, reasonable to suggest that binding of this nuclear basic protein with the membrane phospholipids directly or indirectly affect the conformation of ATPase by electrostatic or steric changes in the membrane.

On the basis of the findings in this and our previous papers(1, 2), the following hypothesis is proposed for the mechanism of the action of clupeine sulfate against *B. subtilis*. Clupeine is adsorbed onto the bacterial cells and results in the loss of viability depending on the quantity adsorbed. This basic protein is probably at first taken up by the cell wall and then by the cell membrane, and the latter appears to be the main site of action. Bactericidal activity results from the rapid disruption of the membrane structure by interaction with membrane phospholipids and then by destroying the membrane functions as secondary effects. These membrane damages are irreversible with minimum inhibitory concentration of clupeine so the membranes are unable to overcome the loss of essential metabolites. According to this hypothesis clupeine may be classified as a cationic membrane-active antibacterial agent.

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